Akirins, highly conserved nuclear proteins, required for NF-κB dependent gene expression in Drosophila and mice

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Abstract

During a genome-wide RNAi screen, we isolated *CG8580* as a gene involved in the innate immune response of Drosophila. *CG8580*, which we named *Akirin*, acts in parallel with the NF-κB transcription factor downstream of the Imd pathway and was required for defense against Gram-negative bacteria. *Akirin* is highly conserved and the human genome contains two homologues, one of which was able to rescue the loss of function phenotype in Drosophila cells. Akirins had a strict nuclear localization. Knockout of both *Akirin* homologues in mice revealed that one had an essential function downstream of Toll-like receptor, tumor necrosis factor and interleukin 1-β (IL-1β) signaling pathways leading to the production of IL-6. Thus, *Akirin* is a conserved nuclear factor required for innate immune responses.

The innate immune system shields all metazoans against invading microorganisms. This well conserved defense mechanism relies on host-pathogen interactions between non-clonally distributed pattern recognition receptors in the host and pathogen-associated molecular patterns in microbes (reviewed in1-4). In contrast, the acquired immune system, based on selection of lymphocytes and their antigen-specific receptors, is specific to vertebrates. Drosophila has become an attractive model organism for the study of the innate immune system due to its well-established genetics, the absence of an acquired immune system and the striking conservation between its immune system and many mammalian

innate immune defenses. One of the hallmarks of the Drosophila defense is the systemic response, which involves the synthesis of small-sized cationic antimicrobial peptides by the fat body, a functional equivalent of the mammalian liver. Two distinct signaling pathways, namely the immune deficiency (Imd) and the Toll pathways control the transcription of the antimicrobial peptide genes (reviewed in[2,4,5]). Fungal or Gram-positive bacterial infections activate the Toll pathway[6]. Briefly, the cytokine-like peptide Spaetzle is cleaved in response to microbial challenge in the open circulatory system of the fly and binds to the transmembrane receptor Toll[7]. The subsequent intracellular cascade leads to the dissociation of the NF-κB family Dorsal-related immunity factor (Dif)[8,9] from its inhibitor, the IκB-like protein Cactus, via the recruitment of the myeloid differentiation factor 88 homologue (MyD88)[10], the adaptor molecule Tube, and the IL-1R-associated kinase (IRAK)-like serine-threonine kinase Pelle[2]. Dif nuclear translocation then activates numerous genes including the antifungal peptide Drosomycin (Drs)[4,6,9]. In contrast, Gram-negative bacterial infection activates the Imd pathway resulting in the expression of antimicrobial peptide genes like Attacin, Cecropin and Diptericin[3,4,11]. Expression of these effector genes requires the signal-dependent cleavage and subsequent nuclear translocation of Relish, another member of the NF-κB family of transcription factors[12-14]. Several genetic screens have identified many players of the Imd pathway and shown striking similarities with components of the mammalian tumour necrosis factor (TNF) pathway[15]. Gram-negative bacterial peptidoglycan (PGN) binds to peptidoglycan recognition protein LC (PGRP-LC) and PGRP-LE, which are the most upstream components of the Imd pathway[16-22]. Imd itself encodes a protein with a death domain (DD) similar to that of the mammalian receptor interacting protein (RIP) that plays an important role in both NF-κB activation and apoptosis[23,24]. Yeast two-hybrid experiments and genetic analysis have demonstrated that Imd forms a complex with the death domain containing adaptor Fadd and the caspase Dredd[25,26]. This upstream protein complex then activates, via a TAK1-binding protein called dTAB2 (ref. 27) and inhibitor of apoptosis protein 2 (IAP2)[28], the Drosophila TGF-β-activated kinase 1 (dTAK1), a member of the MAPKKK kinase family[29]. Both IκB kinase (IKK) beta (DmIKKβ) and gamma (DmIKKγ) are also required downstream of Imd and dTAK1 for Relish activation[30,31]. In mammals, Gram-negative bacteria are sensed by Toll-like receptors (TLRs) that activate, similarly to the Drosophila Imd pathway, an IKK complex and NF-κB. In response to TLR or IL-1R stimulation, MyD88 and IRAKs are recruited to the receptor, and then interact with TNFR-associated factor 6 (TRAF6), which acts as an ubiquitin protein ligase (E3). Subsequently, TRAF6, together with an ubiquitination E2 enzyme complex consisting of UBC13 and UEV1A, catalyzes the formation of a K63-linked polyubiquitin chain on TRAF6 and on IKK-γ-NF-κB essential modulator (NEMO)[32]. A complex comprising TAK1 and the TAK1-binding proteins, TAB1, TAB2 and TAB3, is also recruited to TRAF6 (ref. 33). Upon stimulation by TLR ligands, IκBα is phosphorylated on two serine residues by an IKK complex activated by TAK1. Phosphorylated IκBα is then ubiquitinated and degraded by the proteasome. Liberated NF-κB translocates into the nucleus where it activates the transcription of its target genes.

Despite more than ten years of research since the initial discovery of the Imd mutation, the pathway that took its name is still not fully understood. We undertook a functional genome-

wide RNAi screen in Drosophila cell culture to isolate new components in the Imd pathway. We report here the isolation of *CG8580* (that we renamed *Akirin*) encoding a nuclear protein with no recognizable domain and required for NF-κB dependent transcription. RNAi-mediated knock down of *Akirin* led to impaired Imd pathway signaling and enhanced sensitivity of flies to Gram-negative bacterial infection. Moreover, epistatic analysis allowed us to place the *Akirin* function at the level of the transcription factor itself. As *Akirin* shows striking evolutionary conservation, we generated mice deficient for *Akirin* homologues and demonstrated that one of these mouse *Akirin* homologues was required for NF-κB dependent IL-6 production after TLR agonist, IL-1β or TNF stimulation of embryonic fibroblasts. Drosophila loss of function phenotype could also be restored by expression of the human homologue of *Akirin*. We therefore propose that *Akirin* is an ancient conserved nuclear factor regulating NF-κB dependent transcription.

RESULTS

Identification of Drosophila and mice Akirin homologues

To identify new components of the Imd pathway, we performed a high-throughput RNAi screen with cultured Drosophila S2 hemocyte-like cells (see28, 34 and Methods). Out of 21,306 RNAi probes, several induced a moderate to marked effect on the expression of the Imd pathway-dependent *Attacin* gene activated by an *Escherichia coli* infection. Here we selected *CG8580*, as the corresponding RNAi reduced the induction of Attacin expression by 90%. *CG8580* encodes a putative 201 amino-acid protein with no recognizable domains. Interestingly, two homologues of the *CG8580* sequence are present in zebrafish (*Danio rerio*), xenopus (*Xenopus laevis*), human (*Homo sapiens*) and mouse (*Mus musculus*) databases. Only one copy is present in insects (Apis mellifica, Tenebrio molitor, Anopheles gambiae, Drosophila melanogaster) and in birds (*Gallus gallus*) but none was found in plants, yeast or bacteria. The similarities allow the sequences to be split into discrete groups, one in insects and two in vertebrates (Fig. 1). The conservation is highest for the putative C and N-terminal domains. All sequences show a clear nuclear localization signal (NLS) located between residues 24 and 29 at the N-terminus (Supplementary Fig. 1 online). We renamed the gene *Akirin* (*Akirin1* and *Akirin2* in the case of vertebrates) from the japanese `akiraka ni suru', which means `making things clear'.

Akirins are ubiquitously expressed nuclear proteins

Microarray data in Flybase35 indicate that *DmAkirin* expression is ubiquitous. Similarly, an analysis based on Northern blot with human RNAs, points to almost ubiquitous expression of human *Akirins* (Supplementary Fig. 2 online). To monitor the cellular localization of DmAkirin, we fused the *DmAkirin* coding sequence to a V5-tag and transfected S2 cells. Immunoblot analysis with anti-V5 showed that *DmAkirin* is expressed as a single ~27 kDa protein that is not modified after *E. coli* stimulation (Supplementary Fig. 3 online). Antibody staining of the S2 cells established that DmAkirin has a strict nuclear localization, which was dependent on the presence of the N-terminal NLS (Fig. 2a) and did not change after *E. coli* treatment (data not shown). Similarly, we fused the human *HsAkirin1* and *HsAkirin2* sequences to a Flag-tag and transfected HeLa cells. Antibody staining of the human cells

clearly showed the nuclear localization of HsAkirin1 and HsAkirin2, again dependent on the NLS (Fig. 2b).

Akirin function in Drosophila

To analyze the effects of *DmAkirin* on the Imd pathway, we used an RNAi-mediated knock down strategy in S2 cells. A truncated form of PGRP-LCa (containing only the transmembrane and intracellular segment: TM + Intra) was earlier shown to induce a robust expression of an Attacin-Luciferase (Att-Luc) reporter gene (10,16,17,28 and A.G., unpublished data). Compared to GFP RNAi controls, the induction of the Att-Luc reporter was strongly suppressed by double-stranded RNA (dsRNA) against *DmAkirin* (Fig. 3a,b) in keeping with reduced *DmAkirin* mRNA abundance (Supplementary Fig. 4 online). The degree of reduction was similar to that observed with dsRNA against *Imd* (Fig. 3b). In additional experiments we confirmed the specificity of the suppression by using two different, non-overlapping, dsRNA directed against *DmAkirin* which both gave a strong Att-Luc reduction, comparable to that of the original dsRNA, demonstrating that the suppression is gene-specific (Fig. 3a,c). The Imd pathway responds to Gram-negative bacteria, but the Toll pathway is predominantly activated by Gram-positive bacteria or fungi and culminates in the expression of many genes, including the antifungal peptide Drosomycin (reviewed in5). To address the question whether *DmAkirin* is also involved in the Toll pathway, we transfected an expression construct encoding DmToll ΔLRR, a constitutively active form of DmToll lacking its extracellular leucine-rich repeat (LRR) domain, into S2 cells together with a Drs-Luciferase reporter. As expected, transfection of this constitutively active DmToll ΔLRR resulted in a marked luciferase expression10, which was reduced by dsRNA targeting *Pelle*, a serine-threonine kinase required in the Toll pathway (Fig. 3d and 10). However neither dsRNA against *DmAkirin* nor against *Imd* did affect Drs-Luciferase expression, demonstrating that *DmAkirin* is not involved in the Toll pathway and eliminating the possibility that dsRNA against *DmAkirin* would affect luciferase expression itself.

We next undertook epistatic experiments to analyze the position of *DmAkirin* within the Imd pathway. For this, we transfected S2 cells with expression constructs encoding several genes of the Imd pathway: *PGRP-LE*, *Imd*, *Fadd*, *Dredd* and *Relish*, and monitored Att-Luc expression. Transfection of PGRP-LE, Imd and Relish constructs led to abundant Att-Luc expression (Fig. 4a-c). Fadd transfection led to a dominant-negative effect on *E. coli* induced Att-Luc expression whereas Dredd expression resulted in lower cell viability (data not shown). Importantly, in PGRP-LE transfected S2 cells, the enhanced Att-Luc expression was significantly decreased by cotransfection of either dsRNA against *Imd* or *DmAkirin* (~60% and ~80% respectively, Fig. 3a). Expression of *Imd* also resulted in a robust Att-Luc expression that could be suppressed by both dsRNA against *DmAkirin* indicating that *DmAkirin* acts downstream of *Imd* (Fig. 4b). As expression of Fadd and Dredd in S2 cells did not cause any Att-Luc expression, we decided to transfect the cells with a construct encoding the NF-κB family member Relish, which acts downstream in the Imd pathway. As shown earlier, transfection of full-length Relish only moderately activates the Imd pathway, but a Relish construct deleted for the nucleotides encoding a serine-rich region (ΔS29-S45) leads to a strong Att-Luc expression12. We confirmed this result (Fig. 4c) and further noted that the strong Relish (ΔS29-S45)-dependent reporter gene induction was significantly

suppressed by both dsRNA against *DmAkirin*. This result indicates that *DmAkirin* acts downstream or at the level of Relish (Fig. 4c), which is in agreement with the nuclear localization of DmAkirin. *DmAkirin* expression in S2 cells by itself did not activate the Imd pathway, as monitored by expression of Att-Luc, nor resulted in lower cell viability. Further, it did not exhibit any dominant-negative effect against *E. coli* treatment (data not shown). To ascertain that the expressed *DmAkirin* construct was functional, we set up a rescue experiment. DsRNA against the *DmAkirin* 5′ UTR was synthesized and shown to suppress the activation of the Imd pathway in PGRP-LC transfected cells that actively expressed the reporter gene. However, when the coding sequence of *DmAkirin* devoid of its 5′ UTR (*DmAkirin*-ORF), that is, the target of the dsRNA sequence, was coexpressed in the same cells, Att-Luc expression was rescued such that it was equivalent to wild-type expression. Interestingly, we could also rescue this phenotype with the human orthologue of *DmAkirin*, *HsAkirin2*, clearly indicating that Akirin is functionally and evolutionary conserved (Fig. 4d).

To analyze the *in vivo* function of *DmAkirin*, we first generated null mutants by imprecise excision of EY08097, a P-element located in the first intron of *CG8580*. Out of 430 lines, we isolated seven lines presenting a deletion uncovering the *DmAkirin* gene. However, all deletion lines were homozygous embryonic lethal, indicating that *DmAkirin* is critically required during Drosophila embryonic development (see Discussion). We next tried to knock down *DmAkirin* through a transgenic RNA interference (RNAi) approach[36]. UAS-Akirin RNAi transgenic flies were generated and crossed with different GAL4 drivers (Fig. 5). *DmAkirin* knock down with heat-shock-GAL4 and yolk-GAL4 resulted in reduction of Imd pathway dependent *Diptericin* gene expression after infection with a mix of Gram-positive and Gram-negative bacteria (Fig. 5a,b). Consistent with the result of cell culture data (see Fig. 2d), *Drs* expression was unchanged in these experiments (Fig. 5a,c), indicating that Toll pathway activation does not require *DmAkirin* function. Finally, RNAi-mediated knock down of *DmAkirin* in whole flies led to enhanced sensitivity to Gram-negative bacterial infection (Fig. 5d).

MmAkirin loss of function mouse embryonic fibroblasts (MEFs)

To investigate whether the function of *Akirins* is conserved in the immune response between Drosophila and mammals, we generated mice deficient in either the *MmAkirin1* or the *MmAkirin2* allele. A gene-targeting vector was constructed by placing two *loxP* sites flanking the first coding exon of the *MmAkirin1* gene, and the *loxP* site-flanked *neo*[r] gene into intron 1 of the *MmAkirin1* gene (Supplementary Fig. 5 online). The targeted embryonic stem (ES) cells were transiently transfected with a plasmid encoding the Cre gene to excise the *neo*[r] gene. *MmAkirin1*[flox/+] mice were crossed with a transgenic mouse line expressing Cre in germ cells (CAG-Cre mice). The deletion of the *MmAkirin1* allele was confirmed by Southern blot analysis (Supplementary Fig. 5). *MmAkirin1*^{-/-} mice were born in a Mendelian ratio, grew healthily and did not show gross developmental abnormalities. The expression of *MmAkirin1* mRNA was abrogated in MEFs obtained from *MmAkirin1*^{-/-} mice (Supplementary Fig. 5).

To generate an *MmAkirin2* flox allele, we constructed a targeting vector inserting two *loxP* sites flanking the first coding exon of the mouse *MmAkirin2* gene, with a *loxP* site-flanked *neo*[r] gene (Supplementary Fig. 6 online). The targeted ES cells were transiently transfected with a plasmid encoding the Cre gene to eliminate *neo*[r]. *MmAkirin2*^{+/-} mice were obtained by mating *MmAkirin2*[flox/+] mice with CAG-Cre mice. In contrast to *MmAkirin1*^{-/-}, *MmAkirin2*^{-/-} mice were embryonic lethal, and *MmAkirin2*^{-/-} embryos were not identified even on embryonic day 9.5 (E9.5), indicating that the *MmAkirin2* gene is essential for normal embryonic development in mice (Supplementary Table 1 online). Thus, we generated mouse embryonic fibroblasts (MEFs) from *MmAkirin2*[flox/+] and *MmAkirin2*[flox/-] embryos, and the *loxP*-flanked genomic fragment was excised by retroviral expression of the Cre protein together with the puromycin resistance gene (*Puro*). Puromycin-resistant cells were examined for the expression of *MmAkirin2* by RT-PCR. The expression of *MmAkirin2* was suppressed in Cre-transduced *MmAkirin2*[flox/-] (*MmAkirin2*^{-/-}) MEFs (Supplementary Fig. 6). We could next analyse MEFs specifically lacking *MmAkirin1* or *MmAkirin2*.

Mouse *MmAkirin2* in IL-1β and TLR-mediated responses

Since Drosophila Akirin was critical for the Imd pathway that activates NF-κB via the IKK complex in a similar manner to the mammalian TNF signaling pathway, we hypothesized that mouse Akirins could likewise be involved in TLR-, IL-1β- and TNF-mediated responses. We first examined the cytokine production of *MmAkirin1*^{-/-} MEFs to TLR ligands, IL-1β and TNF stimulation. The production of IL-6 was comparable between wild-type and *MmAkirin1*^{-/-} MEFs in response to all stimuli tested (Fig. 6a). However, when *MmAkirin2*^{-/-} MEFs were stimulated with TLR ligands including MALP-2 and lipopolysaccharide (LPS), IL-1β and TNF, production of IL-6 was severely impaired compared to that of control *MmAkirin2*^{+/-} MEFs (Fig. 6b). Thus, MmAkirin2, but not MmAkirin1, was responsible for the production of IL-6 in response to TLR or IL-1R stimulation.

Then we examined whether MmAkirin2 regulated IL-6 production at the gene expression level. LPS-induced expression of genes encoding IL-6, IP-10, RANTES, or BCL3 two hours after challenge, was severely impaired in *MmAkirin2*^{-/-} MEFs compared to control cells, indicating that MmAkirin2 is critical for the expression of several LPS-inducible genes (Fig. 7a). However, the induction of genes encoding the CXCL1 chemokine KC, IκBα and IκBζ was almost comparable between *MmAkirin2*^{-/-} and control MEFs. The gene induction in response to IL-1β stimulation was similarly impaired in *MmAkirin2*^{-/-} MEFs (Fig. 7b). Thus, MmAkirin2 regulates the expression of a set of LPS- and IL-1β-inducible genes.

As Drosophila Akirin acts together with or downstream of Relish, we next examined the IL-1β- and LPS-dependent activation of NF-κB in *MmAkirin2*^{-/-} MEFs. In response to these stimuli, neither degradation of IκBα nor induction of NF-κB DNA binding was impaired in *MmAkirin2*^{-/-} MEFs (Fig. 8a,b). These data indicate that mouse MmAkirin2 acts together with or downstream of NF-κB in the control of TLR- and IL-1β-inducible gene expression.

DISCUSSION

Akirins represent novel, extremely conserved, nuclear factors that play a role in the metazoan innate immune system. Akirins function during immune and inflammatory responses in Drosophila as well as in mice, most likely at the level of the transcription factor NF-κB. We demonstrate here that *DmAkirin* encodes a nuclear protein that is required downstream in the Imd pathway at the level of the transcription factor Relish in flies. The mammalian counterparts of Akirin have conserved the original function, as mouse Akirin2 was required downstream of TLR, TNF and IL-1β signaling, again at the level of the NF-κB transcription factor for the production of IL-6.

Akirins are highly conserved among different animal species and show two conserved domains, respectively at the N- and C-terminus, separated by a stretch of less conserved residues. The presence of a nuclear localization signal explains the N-terminal conservation and the nuclear staining that we have observed. Akirins are most probably nuclear resident proteins, as we did not see any change in DmAkirin subcellular localization after overexpression or *E. coli* infection.

Drosophila as other insects has only one *Akirin* gene, but the vertebrate genomes that we have analyzed, except for that of birds, contain two copies of the *Akirin* gene (mouse *MmAkirin1* and *MmAkirin2* show 34% and 39% amino acid identity, respectively, with the unique *DmAkirin*). All *Akirin1* genes were similar and segregate from the group containing the *Akirin2* genes, indicating an early duplication event followed by divergence in the evolution of vertebrates. Birds would then have lost secondarily the *Akirin1* gene. The diverging function between Akirin1 and 2 was attested by the contrasting phenotypes of mouse *Akirin* knockouts. *MmAkirin2* was essential for embryogenesis and the cytokine response to TLR and IL-1R stimulation, whereas *MmAkirin1* knockout mice showed no obvious phenotype. *MmAkirin2* would be functionally closer to the single gene in Drosophila as the homozygous null *DmAkirin* mutants show a comparable, mid- to early embryonic lethality. The functional role of *MmAkirin1*, attested by its sequence conservation, is unknown thus far. It is possible that *MmAkirin1* and *MmAkirin2* work redundantly in the regulation of target gene expression in MEFs. Generation of cells lacking both *MmAkirin1* and *MmAkirin2* will help to elucidate the role of *MmAkirin1 in vivo*.

Both Drosophila Akirin and mammalian Akirin2 regulate the expression of a set of genes together with or downstream of NF-κB. These results imply that both Drosophila and mammalian Akirins associate with similar protein(s) for controlling gene expression in the nucleus. Transcription by RNA polymerase II involves the cooperative assembly of an initiation complex that is restrained by the assembly of promoter DNA into nucleosomes and other chromatin structures. Transcription is then modulated by chromatin remodeling cofactors targeting the nucleosomes or general cofactors that associate with the basal transcription machinery. It is unlikely that Akirins regulate transcription by binding directly to DNA, as Akirin sequences show no obvious DNA or RNA binding motif. Following Occam's razor principle, the prediction would be that Akirins act as cofactors to regulate or fine-tune NF-κB transcriptional activity by interacting with components of the chromatin or the transcriptional engine. We tested the hypothesis of a direct interaction of DmAkirin with

DNA or Relish, but we could not immunoprecipitate DNA in ChIP assays with tagged DmAkirin or DmAkirin with a tagged-Relish (data not shown), which means that the postulated associations are either weak or most probably require intermediary components. The notion that Akirins could function to modulate transcriptional factors in several other immune-related processes is strengthened by the report that DmAkirin was found as interacting genetically with pannier, one of the GATA factors involved in heart and blood cell development[37]. Along the same line, following another genome-wide RNAi screen, *DmAkirin* appeared in a list of putative modulators of the Wingless pathway[38] that was recently shown to be involved in the inflammatory response[39]. Taken together these results indicate that Akirins are novel important nuclear cofactors regulating the transcriptional activities of main transactivators. However, further studies are required to clarify how Akirins control gene expression in the Imd and the TLR-IL-1R pathways.

METHODS

DNA constructs

The EST clone of PGRP-LCa (LP06704) was obtained from MRC geneservice. All constructs were subcloned into the *Bam*HI-*Kpn*I sites of the expression vector pPAC42. A PCR fragment of PGRP-LCa was amplified with forward 5'-CCCCGGATCCGATAATTCCCGCCATGCCTTTTAGCAATGAAACG-3' and reverse 5'-GGGGGGTACCTCAGTTCAACGTCTTTCCGAAGAG-3' primers. The PGRP-LE-V5 fragment was obtained from *UAS-PGRP-LE* transgenic flies[21] with the forward 5'-CCCCGG ATCCGATAATTCCCGCCATGTCCGAATCGGGAATC-3' and reverse 5'-GGGGGGTAC CTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGATGGGCTTGCCTTGTTCCT C CTCCTCGATATTG -3' primers. The V5-tagged *DmAkirin* vector was constructed with the forward 5'-CCCCGGATCCGATAATTCCCGCCATGGCCTGTGCAACCCTGAAAC-3' and reverse 5'-GGGGGGTACCTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGA TGGGCTTGCCCGACAGGTAGCTAGGCGCTG-3' primers. The NLS was deleted from V5-tagged *DmAkirin* with the 5'-CTAGACTGGGAGTCGATCAAC-CGTTGCAATCCC TTTGGCCAG-3' primer. The Imd-V5 construct was obtained by exchanging the tag in an Imd-HA construct[26]. *HsAkirin2* was amplified with forward 5'-CCCCGGATCCGATAA TTCCCGCCATGGCGTGCGGAGCCACTCTG-3' and reverse 5'-GGGGGGTACCTCATG AAACATAGCTAGCAGGC-3' primers. Relish constructs were from[12] and TollΔLRR construct from[10]. *UAS-dsDmAkirin* fly stocks were established as in[36] with 5'-GGGGCC GGATCCATGGCCTGTGCAACCC-3' and 5'-GGGGCCGCTAGCTTACGACAGGTAGC-3' primers. N-terminal deletions from *HsAkirin1* and *HsAkirin2* were constructed by PCR using the following primers 5'-AGCTTGGCTCCCCGAAGCGGCGGCGCTGC-3' (Δ20), 5'-AAGCTTCTGCCCGGCCCCACTCCGGGCCTC-3' (Δ30), 5'-AAGCTTTCCCCGAAGCGCAGGCGATGTGCG-3' (Δ20) and 5'-AAGCTTTCGGCGCCC ACC TCGGCCGCTGCC-3' (Δ30), respectively.

Cell culture and transfection assays

Akirin was identified in a large-scale RNAi screen as previously described[28,34]. In brief, 384-well screening plates were pre-spotted with approximately 75nM dsRNA in 5 μl of 1 mM Tris at pH 7. Hemocyte-like Kc167 cells were batch-transfected with an IMD-specific *mtk*-luciferase reporter[28], a truncated form of PGRP-LC, a constitutive expressed *Renilla* luciferase and transferred to dsRNA-containing screening plates. 15,000 cells in 20 μl were dispensed per well and incubated for 1 h before adding serum-containing medium. After 5 days, medium was removed, cells were lysed and both firefly and *Renilla* luciferase activities were determined. Akirin was also identified in IMD-pathway experiments in S2 cells (as described in[28]). S2 cells (Invitrogen and DGRC) were grown at 23 °C in Schneider's medium (Biowest) supplemented with 10% FCS. Cells (1.2×10^6/ml) were transfected in 24 wells plates by calcium phosphate precipitation with 10 μg of *Attacin (Att)-Luciferase* or *Drosomycin (Drs)-Luciferase* reporter vector, 10 μg of an *Actin5C-lacZ* transfection control vector and dsRNAs (1.0 μg/well). After 12-16 h, the cells were washed with PBS and incubated in fresh medium. Cells were stimulated by heat-killed *E. coli* (20~30 bacteria/cell) the next day. After 12-16 h of *E. coli* stimulation, cells were lysed and luciferase activity was measured in a luminometer (BCL Book, Promega) immediately after addition of the substrate (luciferin, Promega). β-galactosidase activity was measured with *O*-nitrophenyl-β-D-galactoside as a substrate, and the values were used to normalize variability in transfection efficiency. For epistatic analysis various amounts (0.001, 0.002, 0.01, 0.02, 0.2 or 0.5 μg/well) of expression vectors were used. For rescue experiments, 0.75 μg of Akirin, 0.025 μg of *PGRP-LC* and 0.25 μg of dsRNAs were transfected. All experiments were done more than two times independently with duplicate wells.

DsRNA preparation

Templates for dsRNAs preparation were PCR-derived fragments between two T7 promoter sequences. Fragments for each gene were: *GFP* (nt 35 - 736, GenBank accession, L29345), *Key* (nt 222 - 744, NCBI accession, NM_079132), *Imd* (nt 331 - 1015, NCBI accession, NM_133166) and *PGRP-LCa*: LP06704 (nt 318 - 1028, NCBI accession, AY119048). Single-stranded RNAs were synthesized with the MEGAscript T7 transcription kit (Ambion). Annealed dsRNAs were ethanol precipitated and dissolved in injection buffer (0.1 mM sodium phosphate, pH 6.8; 5 mM KCl).

Cell staining

S2 cells were fixed with 2% paraformaldehyde (PFA) in PBS for 15 min three days after transfection. Cells were then permeabilized with 0.1% Triton X-100, 1% BSA, PBS for 1 h (PBSBT), incubated overnight with V5 mAb (Invitrogen, 500-fold dilution in PBT: PBS containing 0.1 % Tween 20), washed and incubated with FITC anti-mouse IgG (500-fold dilution in PBS, Jackson ImmunoResearch). Cells were stained with DAPI in PBS to visualize nuclei and observed with a Zeiss Axioskop 2 microscope.

Microbial infections, Survival experiments and Northern blot analysis

We used the following bacterial strains: *E. coli* (1106), *Micrococcus luteus* (*M. luteus*, CIP A270) and *Agrobacterium tumefasciens*. Survival experiments were carried out as described

previously43. For Northern blot analysis, flies were challenged with a thin tungsten needle previously dipped into a concentrated culture of mixed Gram-positive (*M. luteus*) and Gram-negative (*E. coli*) bacteria. After 6 h (for *Dip*) or 24 h (for *Drs*), flies were collected. MEFs (1×10^6) were stimulated with 10 ng/ml of IL-1β or 10 mg/ml of LPS for 2 or 4 h. Total RNA was extracted with TRIzol (Invitrogen). RNA (20 μg for flies; 10 μg for MEFs) was electrophoresed, transferred to nylon membrane (Hybond N+; Amersham Pharmacia Biotech) and hybridized with a specific cDNA probe for *Dip*, *Drs*, *Il6*, *Nfkbia*, *Nfkbiz, Bcl3*, *Ccl5, Cxcl1* and *Cxcl10*. The same membrane was stripped and rehybridized with an *Rp49* (flies) or an *Actb* cDNA probe as internal control. Signals were quantified with BAS 2000 Image Analyzer (Fuji Photo Film Co.).

Fly strains and crosses

Flies were grown on standard medium at 25 °C. Drosophila Gal4 driver stocks are described in44. RelishE20 and white1118 were used as Imd pathway mutant and wild-type control respectively. Transgenic w^{1118}; +/+; *UAS-dsDmAkirin/TM3* males were crossed with either w^{1118}; *heat-shock (hs)-GAL4/CyO; +/+*, or w^{1118}; +/+; *yolk-GAL4* females and the progeny was kept at 29 °C.

Establishment of *Akirin2$^{-/-}$* MEFs

MEFs were obtained from E13.5 *Akirn2*flox/+ or *Akirn2*flox/- embryos. For excision of the floxed genomic fragment containing exon 1, the MEFs were infected with retrovirus expressing Cre protein together with puromycin-resistant gene product. At 24 h after infection, 3 mg/ml of puromycin (Invivogen) was added and selected for 72 h. Then the MEFs were used for analysis. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).

Measurement of IL-6 production

MEFs (2×10^4) were stimulated with 0.1 and 1 μg/ml of recombinant mouse IL-1β (R&D Systems), 10 μg/ml of LPS (Sigma), 1 and 10 nM of MALP-2 or 1 and 10 ng/ml of recombinant mouse TNF (R&D Systems) for 24 h. Culture supernatants were collected and IL-6 concentrations measured with the ELISA kit (R&D Systems).

Immunoblot analysis

MEFs (2×10^6) pre-incubated in FBS-free medium for 1 h were stimulated with 10 ng/ml of IL-1β in FBS-free medium or 10 mg/ml of LPS in medium containing 0.3% FBS for various periods. MEFs were then lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and a protease inhibitor cocktail (Roche). Lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (BioRad). Membranes were probed with antibodies and visualized with an enhanced chemiluminescence system (Perkin-Elmer). Polyclonal antibody to IκBα (anti-IκBα and HRP-conjugated monoclonal anti-β-tubulin (clone D-10) were purchased from Santa Cruz. Monoclonal anti-phosho-p65 (Ser536) (clone 7F1) was purchased from Cell Signaling.

Electrophoretic mobility-shift assay (EMSA)

MEFs (2×10^6) pre-incubated in FBS-free medium for 1 h were stimulated with 10 ng/ml of IL-1µ in FBS-free medium or 10 mg/ml of LPS in medium containing 0.3% FBS for various periods. Nuclear extracts were purified from cells, incubated with a probe specific for NF-κB DNA-binding sites, separated by electrophoresis and visualized by autoradiography.

Statistical analysis

Mean values and standard deviations were calculated with Excel software (Microsoft).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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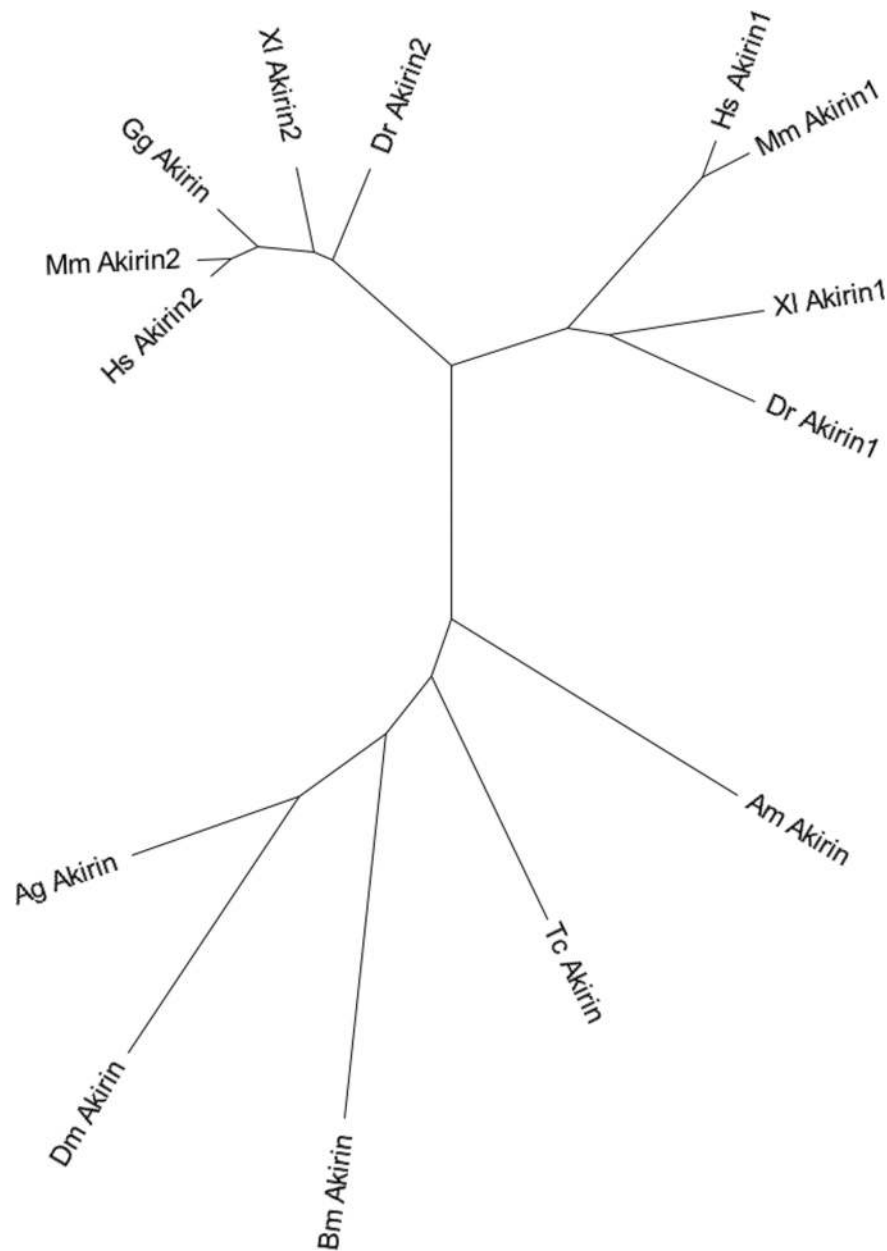
Figure 1. Akirins are highly conserved

Sequences were retrieved by homology search with BLAST using the *Drosophila melanogaster* CG8580 from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), except for *Bombyx mori* for which the silkbase was used (http://papilio.ab.a.u-tokyo.ac.jp/silkbase/). The sequences were as follows: Dm_Akirin for *Drosophila melanogaster* Akirin (NP_648113), Ag_Akirin for *Anopheles gambiae* Akirin (XP_308938, modified), Bm_Akirin for *Bombyx mori* Akirin (wdS20131), Am_Akirin for *Apis mellifera* Akirin (XP_395252), Tc_Akirin for *Tribolium castaneum* Akirin (XP_971340), Gg_Akirin for *Gallus gallus* Akirin (XP_419845), Hs_Akirin1 for *Homo sapiens* Akirin1 (NP_078871), Mm_Akirin1 for *Mus musculus* Akirin1 (NP_075912), Xl_Akirin1 for *Xenopus laevis* Akirin1 (AAH72831), Dr_Akirin1 for *Danio rerio* Akirin1 (NP_001007187), Hs_Akirin2

for *Homo sapiens* Akirin2 (NP_060534), Mm_Akirin2 for *Mus musculus* Akirin2 (NP_001007590), Xl_Akirin2 for *Xenopus laevis* Akirin2 (AAH72831) and Dr_Akirin2 for *Danio rerio* Akirin2 (NP_998707). Sequences were aligned with MULTALIN40 (see Fig. S1). In vertebrates, similarity splits *Akirins* into two groups that we have numbered 1 and 2, with *Akirin2s* more closely related to invertebrate *Akirins*. Subsequent assembly into a majority consensus minimum evolution bootstrap tree was made using the MEGA3 software41.
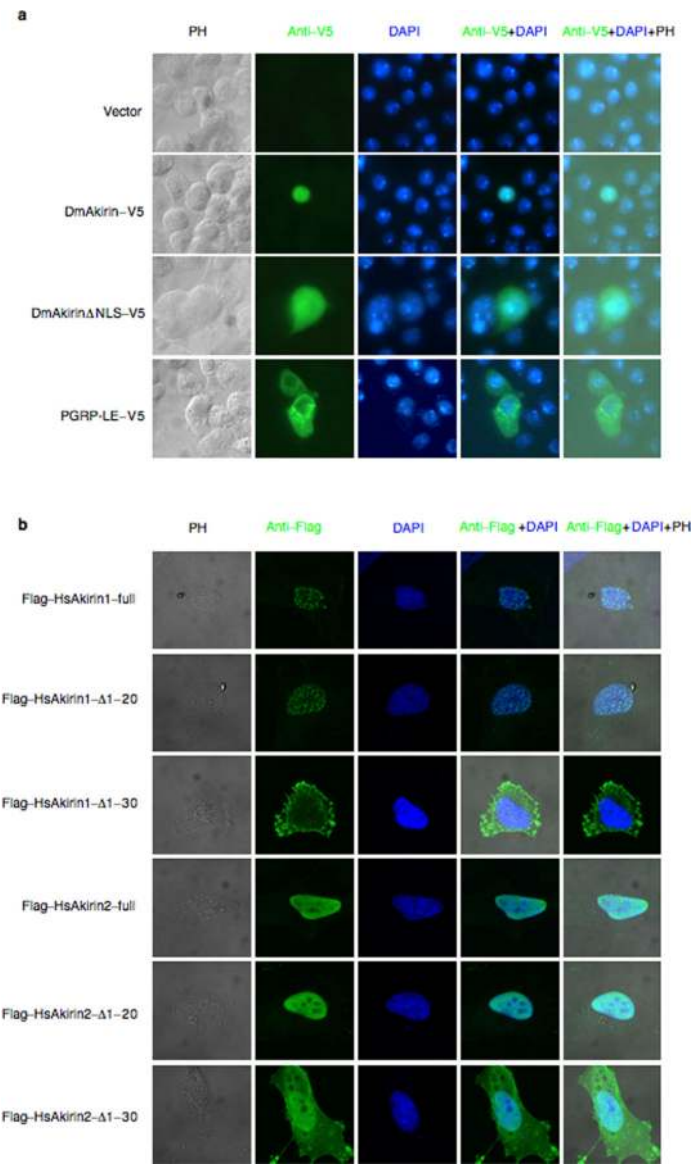
Figure 2. Nuclear localization of Akirins

(a) S2 cells were transfected with V5-tagged, *DmAkirin,* NLS-deleted *DmAkirin* or *PGRP-LE*. Cells transfected with an empty vector were used as control. For cell staining, nuclei were visualised using DAPI (Blue). DmAkirin, NLS-deleted DmAkirin and PGRP-LE were revealed by a V5 antibody (Green). The merged fields observed in phase contrast (PH) clearly show a nuclear localisation of DmAkirin (Anti-V5+DAPI+PH) compared to the cytoplasmic localization of PGRP-LE. This nuclear localisation is abrogated when the NLS is deleted from DmAkirin. These results are representative of 3 independent experiments. (b) Hela cells were transfected with Flag-tagged full length or N-terminally deleted (aa 1 to 20 or aa 1 to 30) *HsAkirin1* or *HsAkirin2*. For cell staining, nuclei were visualised using DAPI (Blue) and HsAkirins were revealed by an Anti-Flag Antibody (Green). The merged fields, shown in phase contrast (Anti-Flag+DAPI+PH), demonstrate the NLS-dependent (aa 20 to 30, see Fig. S1) nuclear localisation for both HsAkirin1 and HsAkirin2.
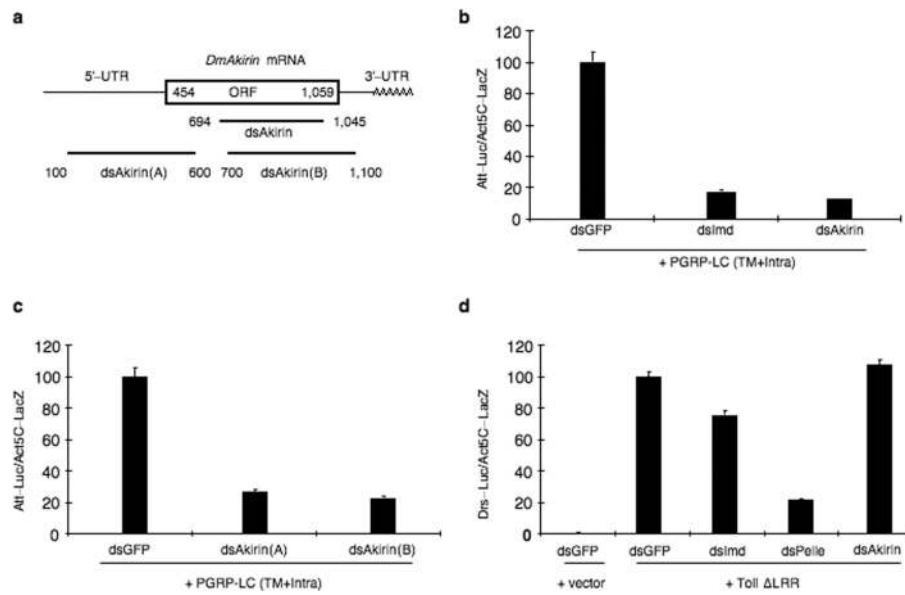
Figure 3. RNAi knock down effect of *DmAkirin* on the activation of the Imd and Toll pathways in Drosophila S2 cells

(a) Structure of *DmAkirin* mRNA (cDNA: LD26817) and of the dsRNAs against *DmAkirin*. An original dsRNA (dsAkirin: HFA 11000) covering nucleotides 694-1045 was used for the screen. Two additional dsRNA (dsAkirins(A) and (B)) covering nucleotides 100-600 and 700-1100 respectively were synthesized. Each bar represents the mean of three independent experiments. Error bars are Standard Deviation (SD).

(b) S2 cells cotransfected with a PGRP-LC (TM+Intra) expression vector, Attacin-Luciferase (Att-Luc) and Actin5C (Act5C)-LacZ reporter genes expression vectors, were treated with either dsRNA against *GFP* (dsGFP), *Imd* (dsImd) or *DmAkirin* (dsAkirin). β-gal and Luciferase activities were measured to normalize transfection efficiency and activation of the Imd pathway respectively. PGRP-LC transfected cells constitutively express the Att-Luc reporter. This expression level is reduced by dsAkirin compared to dsGFP control and is similar to that observed in dsImd treated cells.

(c) Both dsAkirin(A) and (B) suppressed the Att-Luc induction in the same manner as the original dsAkirin.

(d) To monitor the effect of dsAkirin on the activation of the Toll pathway, S2 cells cotransfected with a TollΔLRR expression vector and a Drosomycin-Luciferase (Drs-Luc) reporter gene were treated with either dsRNA against *GFP* (dsGFP), *Pelle* (dsPelle) or *DmAkirin* (dsAkirin). In contrast to dsPelle treated cells, Drs-Luc reporter gene expression induced by the constitutively active TollΔLRR form is not changed in dsImd and dsAkirin treated cells compared to dsGFP controls.
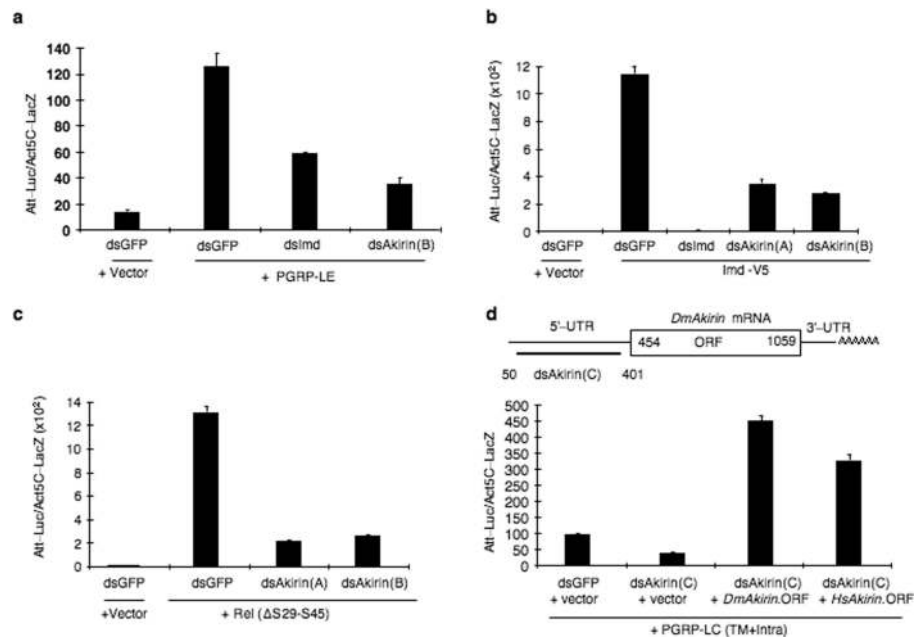
**Figure 4. Epistatic Analysis of DmAkirin position within the Imd pathway**
Several signal modifiers of the Imd pathway, PGRP-LE-V5 (a), Imd-V5 (b), Rel (ΔS29-S45) a serine rich region deleted Relish (c) and PGRP-LC (TM+Intra) (d) were expressed in S2 cells. Cell transfected with the vector alone were used as control. Cells were treated with dsGFP, dsImd or different dsAkirins. Imd pathway activation was monitored with the Att-Luc reporter gene. Each bar represents the mean of three independent experiments. Error bars are (SD).

(a) Att-Luc reporter gene expression induced by PGRP-LE transfection is significantly reduced after either dsImd or dsAkirin(B) transfection.

(b) Att-Luc reporter gene expression induced by Imd expression is highly reduced either by dsImd, dsAkirin(A) or dsAkirin(B).

(c) Att-Luc reporter gene expression induced by Rel(ΔS29-S45) expression is reduced by either dsAkirin(A) or dsAkirin(B).

(d) In order to set up a rescue experiment for *DmAkirin* function in S2 cells, an additional dsRNA targeting the 5'UTR of *DmAkirin* (dsAkirin(C)) was designed. Att-Luc expression induced by PGRP-LC (TM+Intra) expression was highly reduced by dsAkirin(C) and was rescued by the coexpression in the same cells of the coding sequence (ORF) of *DmAkirin* or *HsAkirin2*.
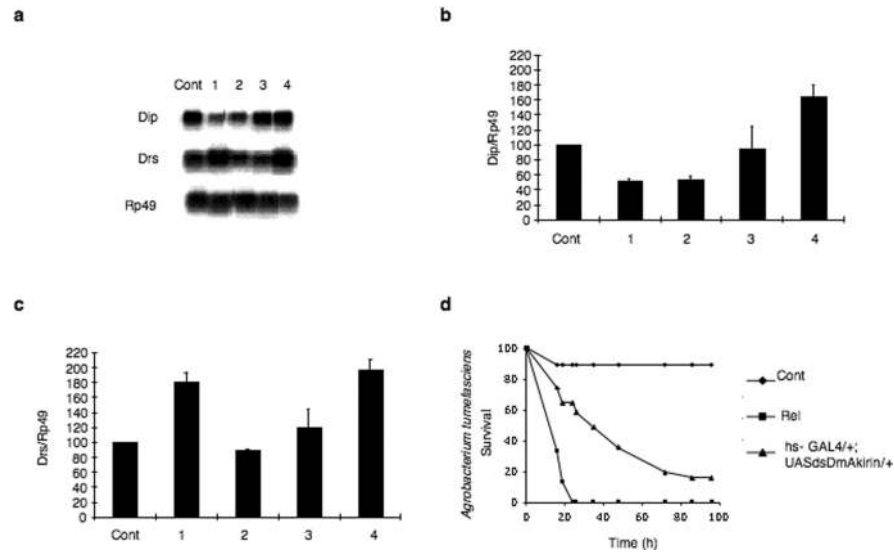
Figure 5. *In vivo* **function of DmAkirin**

UAS-dsDmAkirin transgenic flies were crossed with flies carrying either heat-shock- (hs) or yolk-Gal4 drivers. Progenies were infected with a mixture of Gram-positive (*Micrococcus luteus*) and Gram-negative (*Escherichia coli*) bacteria. The Imd and Toll pathway activations were monitored by Northern blot analysis of the *Diptericin* (*Dip*) and *Drosomycin* (*Drs*) messengers expression. The *Rp49* messenger was used as loading control (a). Quantification of *Dip* (b) and *Drs* (c) normalized with *Rp49*. 1: *hs-GAL4/+;UAS-dsDmAkirin/+*, 2: *yolk-GAL4/+; UAS-dsDmAkirin/+* (females), 3: *CyO/+; UAS-dsDmAkirin/+*, 4: *yolk-GAL4/+;UAS-dsDmAkirin/+* (males). Homozygous *white*[1118] flies were used as control (cont). Each bar represents the mean of three indepent experiments. Error bars are SD.

(d) *UAS-dsDmAkirin* transgenic flies were crossed to *hs-GAL4* driver transgenic flies. After heat-shock, progenies (25 adult flies) were infected with a Gram-negative bacterium (*Agrobacterium tumefasciens*) and surviving flies were counted every 24 hours. The Imd pathway mutant flies, Relish[E20] (Rel), are highly sensitive to this bacterial infection. Compared to control (*white*[1118]) flies, *DmAkirin* knocked down flies presented an increased sensitivity to infection. These results are representative of three independent experiments.
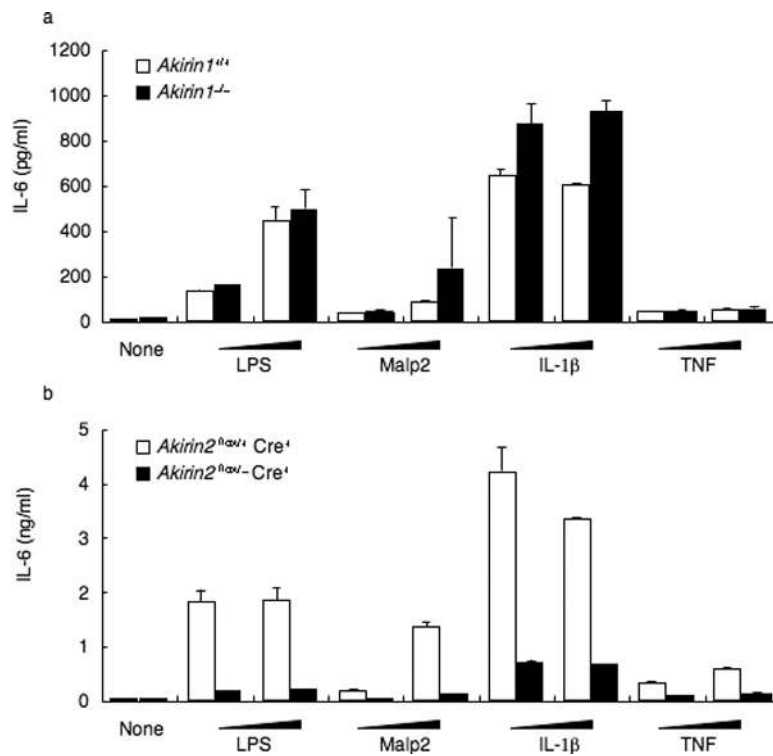
Figure 6. TLR-, IL-1β- and TNF-induced IL-6 production in *Akirin1*^{-/-} and *Akirin2*^{-/-} mouse embryonic fibroblasts (MEFs)

Akirin1^{+/+} and *Akirin1*^{-/-} MEFs (a) and Cre-transduced *Akirin2*[flox/+] and *Akirin2*[flox/-] MEFs (b) were stimulated with increasing concentrations of LPS (1, 10μg/ml), MALP-2 (1, 10 nM), IL-1β (1, 10 ng/ml) and TNF (1, 10 ng/ml) for 24 h. Concentrations of IL-6 in culture supernatant were determined by ELISA. Unlike *Akirin*^{-/-} MEFs, the IL-6 induced production in *Akirin2*^{-/-} MEFs is reduced compared to corresponding wild-type control cells. Each bar represents the mean of three independent experiments. Error bars are SD.
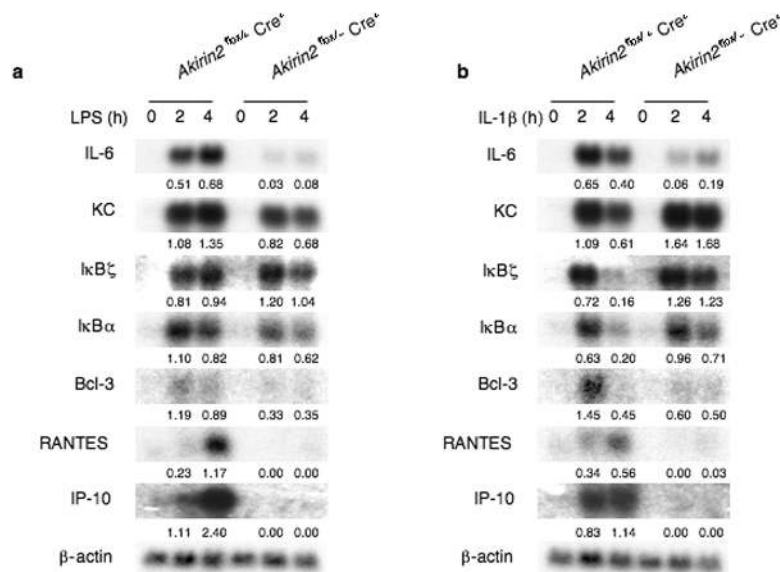
Figure 7. LPS- and IL-1β-induced gene expression in *Akirin2*^{-/-} MEFs

Cre-transduced *Akirin2*^{flox/+} and *Akirin2*^{flox/-} MEFs were stimulated with LPS (10 μg/ml) (a) and IL-1β (10 ng/ml) (b) for 2 and 4 h. Total RNA was extracted from the cells, and subjected to Northern blot analysis for the expression of IL-6, KC, IκBξ, IκBξ, BCL3, RANTES and IP-10. The β-actin messenger is used as loading control. Signals were quantified by NIH image and values indicated as relative density compared to the corresponding loading control. The expression of several LPS- and IL-β-inducible genes is reduced in *Akirin2* deficient MEFs compared to wild-type control cells. These results are representative of three independent experiments.
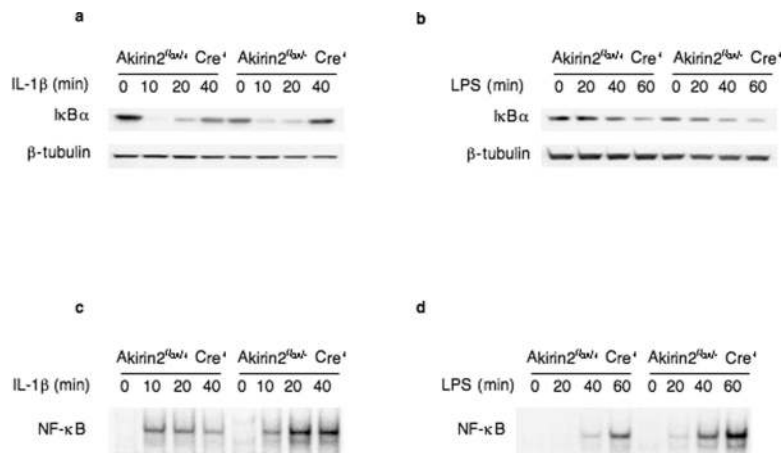
Figure 8. LPS- and IL-1β-induced activation of NF-κB in *Akirin2*^{-/-} MEFs

Cre-transduced *Akirin2*[flox/+] and *Akirin2*[flox/-] MEFs were stimulated with IL-1β (10 ng/ml) (a and c) and LPS (10 µg/ml) (b and d) for the indicated periods. IκBα expression in the whole cell lysates was analyzed by immunoblotting (a and b). NF-κB-DNA binding activity in the nuclear extracts was determined by EMSA (c and d). IκBα degradation and NF-κB-DNA binding activity are similar in wild-type and *Akirin2* deficient cells upon IL-β and LPS stimulations. These results are representative of three independent experiments.